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Early Detection of Asian Soybean Rust Using PCR

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Abstract

Early detection of Asian soybean rust (ASR) is essential to help producers minimize the impact of this serious disease. DNA from ASR-infected soybean plants was used to compare conventional and real-time ASR-specific PCR assays at seven laboratories in the United States. Soybean plants were inoculated with four concentrations of rust spores to establish different levels of infection within the plants. Plant tissue was then harvested at 7 time points over the course of 12 days, and DNA was extracted using two commercially available kits. DNA extracted from soybean plants inoculated with different spore concentrations was tested for ASR using conventional or real-time PCR assays. ASR was consistently detected at 6 days following inoculation with both the conventional and real-time PCR assays. This study demonstrates the ability to reliably detect ASR in soybean before the presence of readily visible symptoms. It also demonstrates the reproducibility of the PCR assay across different real-time PCR platforms at multiple laboratories when the assays were performed in multiple diagnostic laboratories.

Introduction

Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* was confirmed for the first time in the continental United States on 10 November 2004 (11). Historically this species was limited to Australasia, the islands of Japan, the Philippines, and Taiwan. Another soybean rust species, *P. meibomiae,* is much less aggressive and found in Central and South America and the Caribbean (7). The detection of ASR in Africa in 1996 (3) and South America in 2001 (9) revealed *P. pachyrhizi*'s ability to spread beyond its traditional geographic range, thus becoming a potential threat to U.S. agriculture. Unlike other rusts, ASR can naturally infect a very broad range of plant species including 31 species in 17 genera in the legume family (12). In addition, 60 plant species belonging to 26 other genera have been infected under experimentally controlled conditions (10,12).

In general, environmental conditions that promote good growth and full canopy development of the soybean crop are also conducive for the development of ASR; temperatures in the range of 15 to 28°C and moisture in the form of rain or dew for 6 to 12 h are optimal (6). Temperature and moisture conditions typically found in the North Central region of the U.S. are well within the ranges that would result in severe ASR epidemics. Yield reductions of up to 70 to 80% have been reported in Asia, and there is great concern that significant crop losses could occur in the United States (1). It has been estimated that yield losses could exceed 10% in most of the United States and up to 50% in the Mississippi Delta and southeastern states (13).

There is no resistance to ASR in any commercially available soybean cultivar (7). Fungicides exist that will slow the spread of the pathogen, but they require additional input costs for growers (8). It is critical that sensitive and rapid diagnostic methods are available to detect soybean rust at the earliest stage possible, thereby allowing producers to effectively time the application of fungicides that limit the impact and spread of the disease.

The objectives of this study were to test the efficacy of a previously described PCR assay to detect the presence of ASR at different stages of infection and to test the uniformity of the PCR assay across different PCR platforms at multiple diagnostic laboratories.

Plant Inoculations, Sample Collection, and DNA Extractions

All inoculations, sample collections, and DNA extractions were conducted in the USDA-ARS Plant Pathogen Containment greenhouse facility at the Foreign Disease-Weed Science Research Unit (FDWSRU), Ft. Detrick, MD (5). Urediniospores were propagated on soybean 'Williams' inoculated with *P. pachyrhizi* isolate Taiwan 72-1 by the method described previously (2). Urediniospores were suspended in sterile distilled water containing 0.01% Tween 20 (vol/vol) at concentrations of 12,500 spores/ml, 25,000 spores/ml, 50,000 spores/ml, and 100,000 spores/ml. The leaves of soybean plants were spray-inoculated with 2.5 ml of inoculum per plant using an atomizer attached to an air compressor. Fourteen plants at the fifth trifoliolate stage (V5) were inoculated at each of the four spore concentrations. Four control plants were sprayed with sterile Tween water containing no urediniospores. The plants were incubated overnight in a dew chamber at 20°C then transferred to a greenhouse where the temperatures ranged from 18 to 25°C.

Two trifoliolate leaves (six "leaflets") were collected from each of two plants at 1, 3, 6, 7, 10, 11, and 12 days post inoculation (dpi) for each of the four inoculum concentrations. Two trifoliolate leaves were collected from each of two control plants at 1 and 7 dpi. The leaf tissue for each sample time and inoculum concentration was pooled together into plastic freezer bags and stored at -80°C prior to DNA extraction.

Frozen leaf samples were manually crushed, and 100 mg of plant material was transferred to a sterile 2-ml screw-cap tube containing two 5-mm glass beads. Capped sample tubes were placed into liquid nitrogen for 30 s, transferred to the MiniBeadbeater-8 (Biospec Products, Bartlesville, OK) and homogenized for 30 s at maximum speed. Freezing and homogenization was repeated twice. DNA extractions were done using the DNeasy Plant mini kit and the QIAamp DNA Stool mini kit (both from QIAGEN, Inc., Valencia, CA) following the manufacturer's protocol for each kit. DNA extracts were distributed to the following participating laboratories: Cornell University (Cornell), Kansas State University (KSU), Michigan State University (MSU), the University of Florida (UF), the University of Tennessee (UT), and the USDA-APHIS PPQ CPHST National Plant Germplasm and Biotechnology Laboratory (NPGBL).

PCR Evaluations

Extracted DNA was diluted 1:2 and 1:20 with sterile distilled water, and 1-µl of diluted DNA were used for both the conventional and real-time PCR assays. Each sample was tested once at each location. Oligonucleotide primers specific for *P. pachyrhizi* were used to perform conventional PCR assays on all four inoculum concentrations at UT and MSU as previously described (2). PCR

products were resolved on a 1.5% agarose gel run at 100 V, stained with ethidium bromide, and assessed visually. The expected 141-bp ASR-specific PCR product was clearly visible by 6 dpi with DNA extracted using both kits from soybean leaves inoculated at the highest spore inoculum concentration (100,000 spores/ml), and faint bands were also visible at the two lower spore inoculum concentrations (12,500 and 25,000 spores/ml) (Fig. 1).

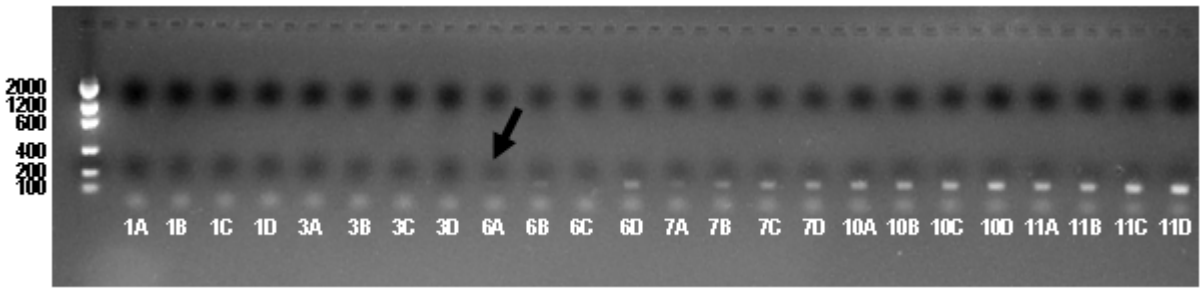
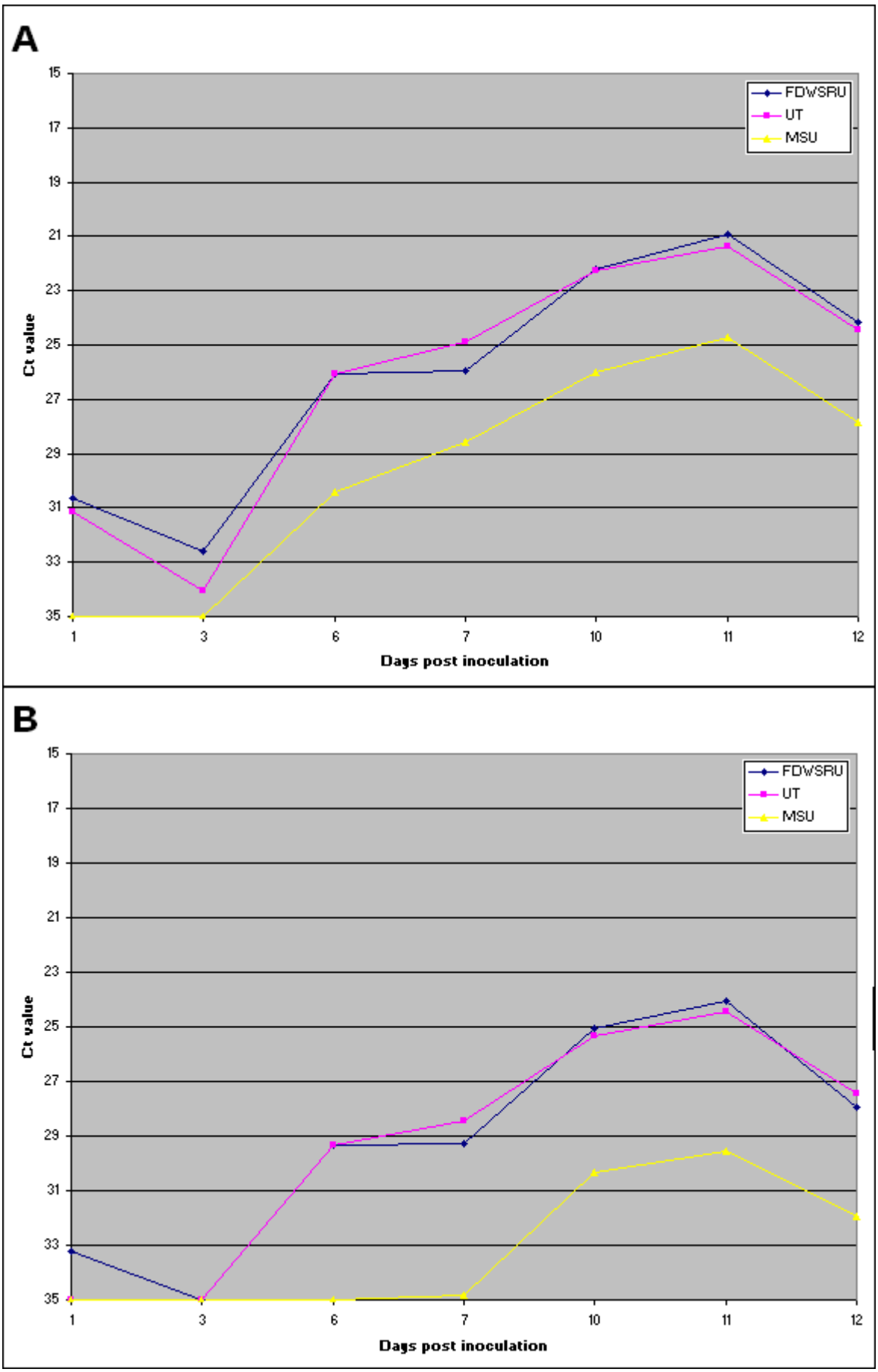


Fig. 1. Agarose gel of conventional PCR performed at the University of Tennessee using DNA extracted from soybean leaves infected with *Phakopsora pachyrhizi* and the ASR-specific primers. The first lane contains a low mass DNA ladder (Invitrogen, Carlsbad, CA) with fragment sizes of 100, 200, 400, 600, 1200, and 2000 bp. Sample numbers correspond to the number of days post infection (dpi), and the letters indicate inoculum spore concentrations: A = 12,500/ml; B = 25,000/ml; C = 50,000/ml; and D = 100,000/ml. The arrow indicates the first clearly visible PCR amplification product.

Real-time PCR assays using the ABI sequence detection system (ABI Prism 7000 or 7700; Applied Biosystems, Inc., Foster City, CA) were performed on samples from three of the four inoculum concentrations (12,500, 25,000, and 100,000 spores/ml) at the FDWSRU, MSU, and UT as previously described (2). The ABI Prism sequence detection software calculated the cycle threshold (Ct) values for each PCR sample by determining the point in time (PCR cycle number) at which the reporter fluorescence exceeds background. Noninoculated plant controls from 1 and 7 dpi tested negative for ASR on the ABI platform. ASR was detected from plants inoculated at all three spore concentrations at 6 dpi at all three laboratories using DNA extracted with both of the commercial DNA kits. The 1:2 DNA dilutions produced a stronger positive signal (lower Ct value) for all the spore treatments compared to the 1:20 DNA dilutions that were generally 3 Ct values higher (Figs. 2 and 3). While the samples tested at MSU had higher Ct values than at UT or the FDWSRU, the overall amplification profile was similar.

Fig. 2 (next 3 pages). Real-time PCR of soybean leaves infected with *Phakopsora pachyrhizi* performed using the ABI sequence detection system. The vertical axis shows the cycle threshold (Ct) value (the PCR cycle when fluorescence exceeds background) that indicates a positive identification. The horizontal axis indicates the number of days after inoculation at which each sample was collected. DNA was extracted from inoculated leaves using the DNeasy Plant Mini kit (QIAGEN) and tested at two dilutions at the inoculum concentration as follows: (**A**) 1:2 DNA dilution, inoculum at 12,500 spores/ml; (**B**) 1:20 DNA dilution, inoculum at 12,500 spores/ml; (**C**) 1:2 DNA dilution, inoculum at 25,000 spores/ml; (**D**) 1:20 DNA dilution, inoculum at 25,000 spores/ml; (**E**) 1:2 DNA dilution, inoculum at 100,000spores/ml; and (**F**) 1:20 DNA dilution, inoculum at 100,000 spores/ml.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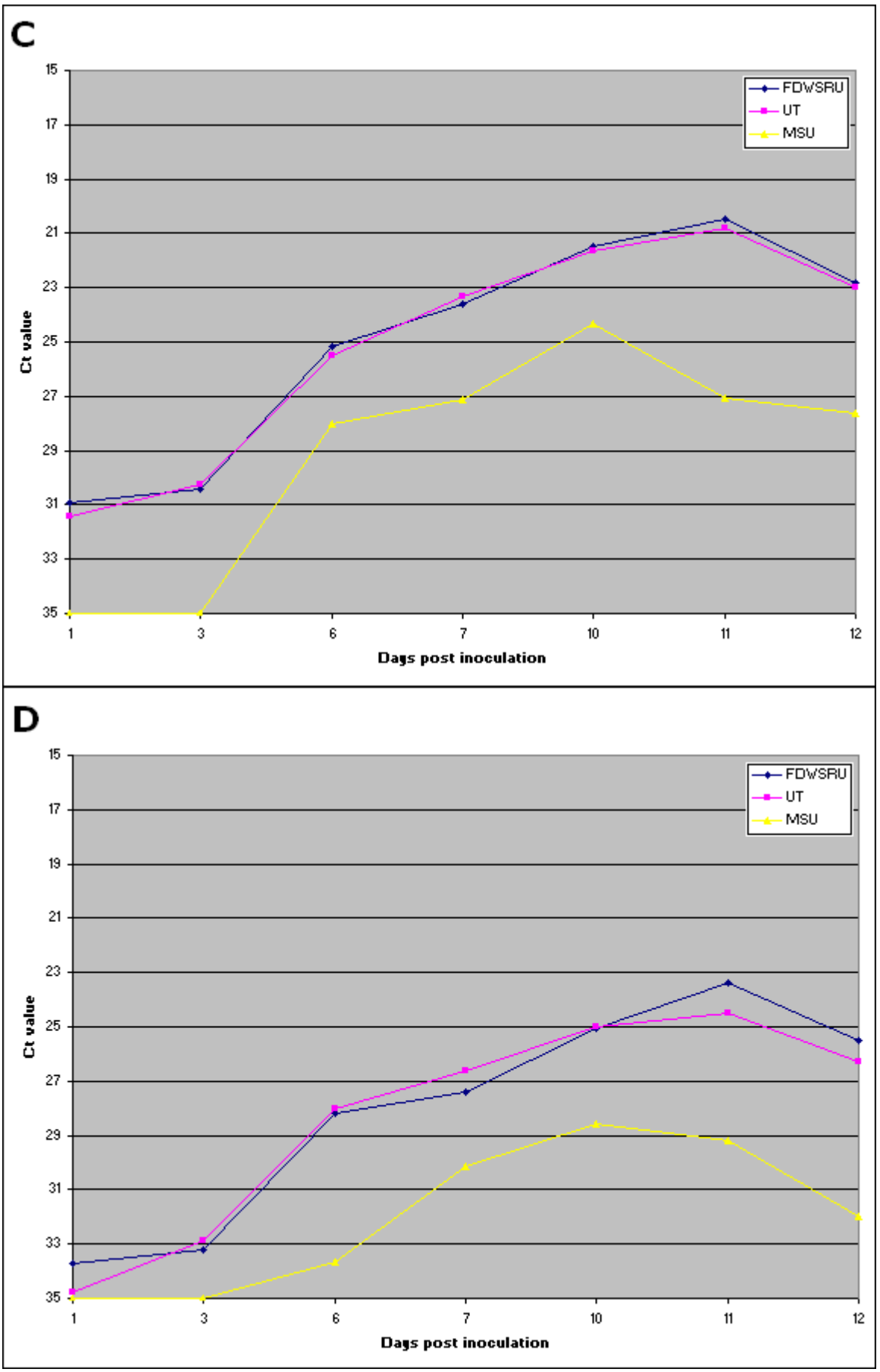
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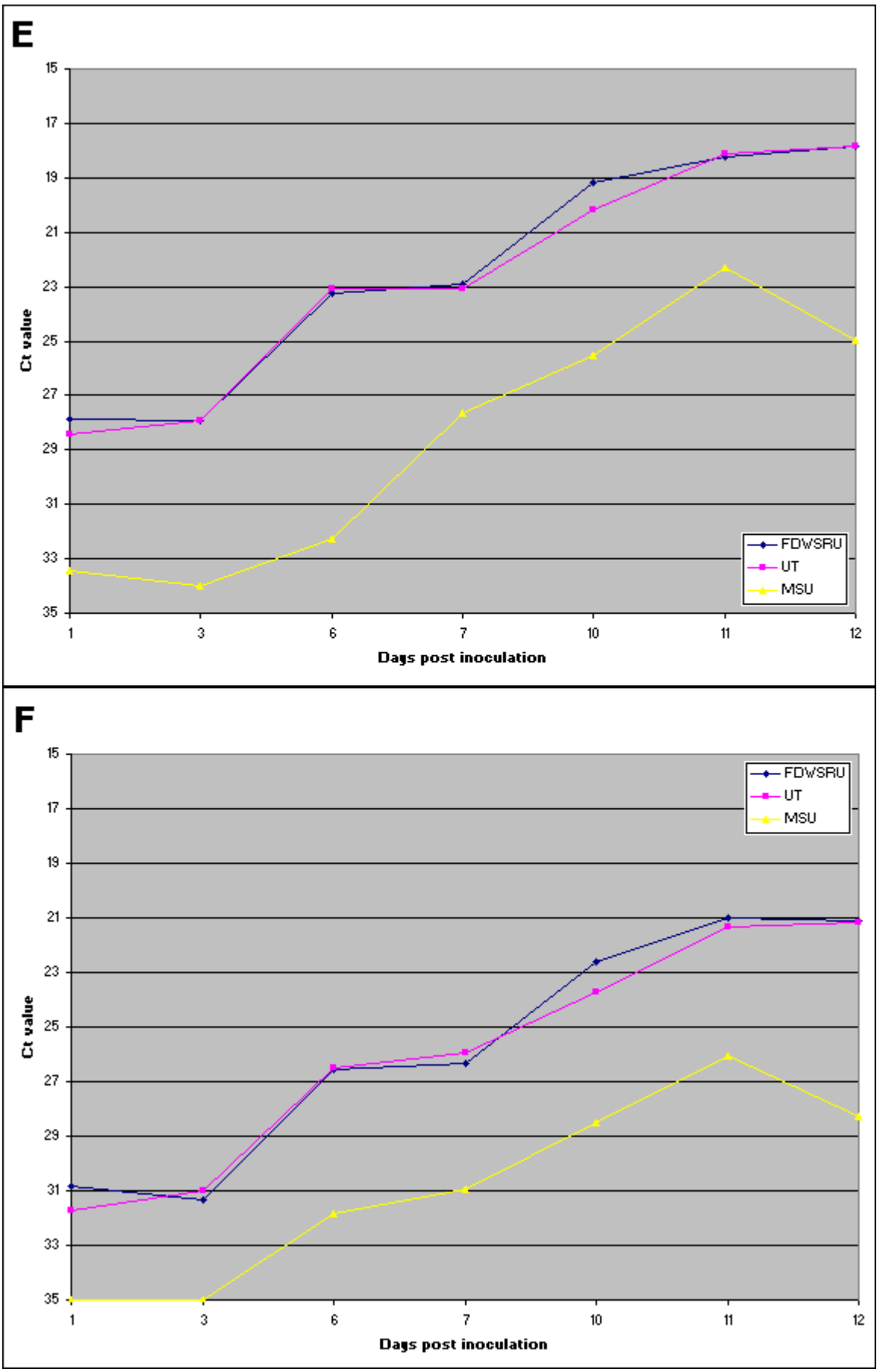
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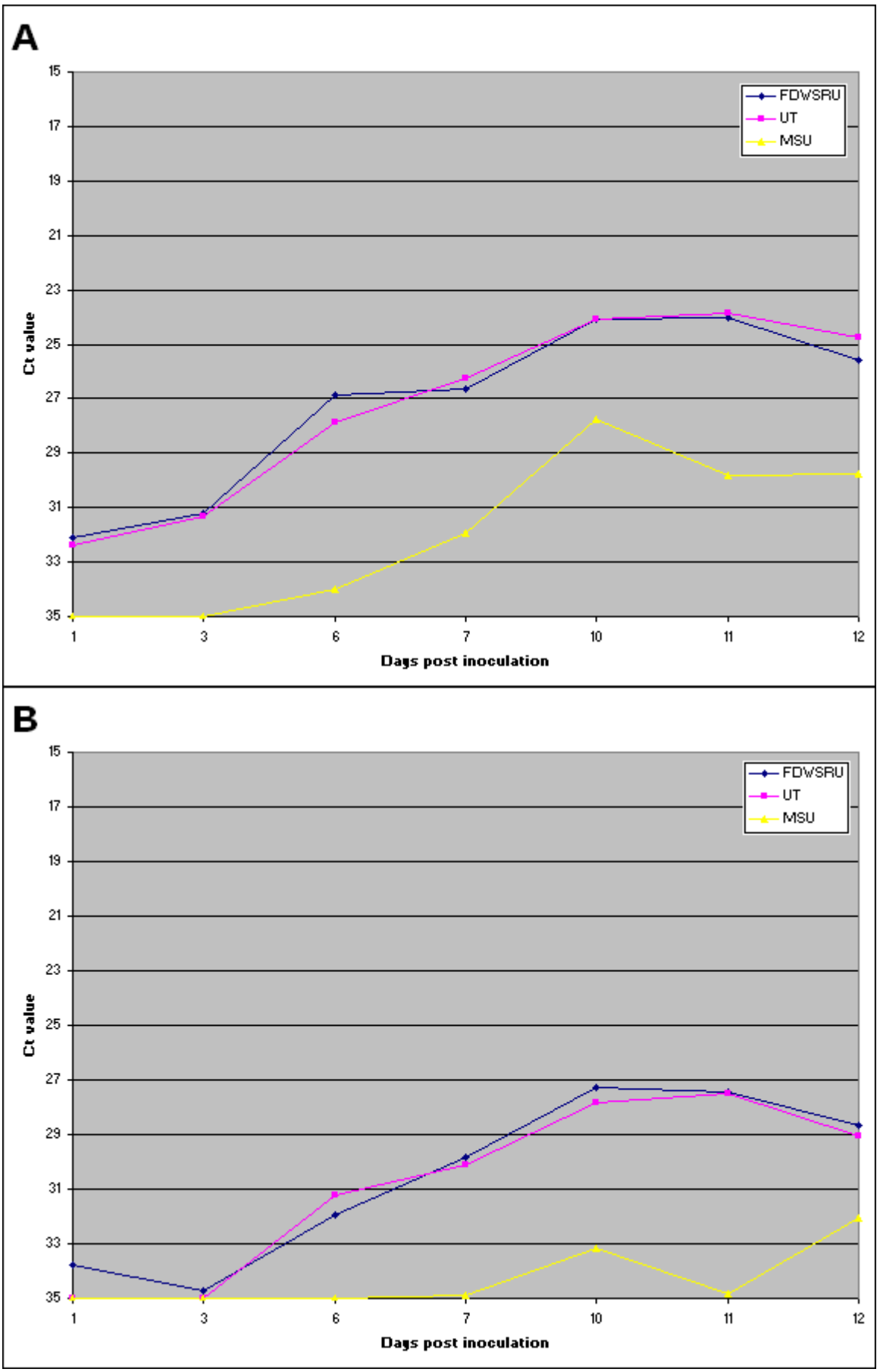
Fig. 3 (next 3 pages). Real-time PCR of soybean leaves infected with *Phakopsora pachyrhizi* performed using the ABI sequence detection system. The vertical axis shows the cycle threshold (Ct) value (the PCR cycle when fluorescence exceeds background) that indicates a positive identification. The horizontal axis indicates the number of days after inoculation at which each sample was collected. DNA was extracted from inoculated leaves using the QIAamp DNA Stool mini kit (QIAGEN) and tested at two dilutions at the inoculum concentration as follows: (**A**) 1:2 DNA dilution, inoculum at 12,500 spores/ml; (**B**) 1:20 DNA dilution, inoculum at 12,500 spores/ml; (**C**) 1:2 DNA dilution, inoculum at 25,000 spores/ml; (**D**) 1:20 DNA dilution, inoculum at 25,000 spores/ml; (**E**) 1:2 DNA dilution, inoculum at 100,000spores/ml; and (**F**) 1:20 DNA dilution, inoculum at 100,000 spores/ml.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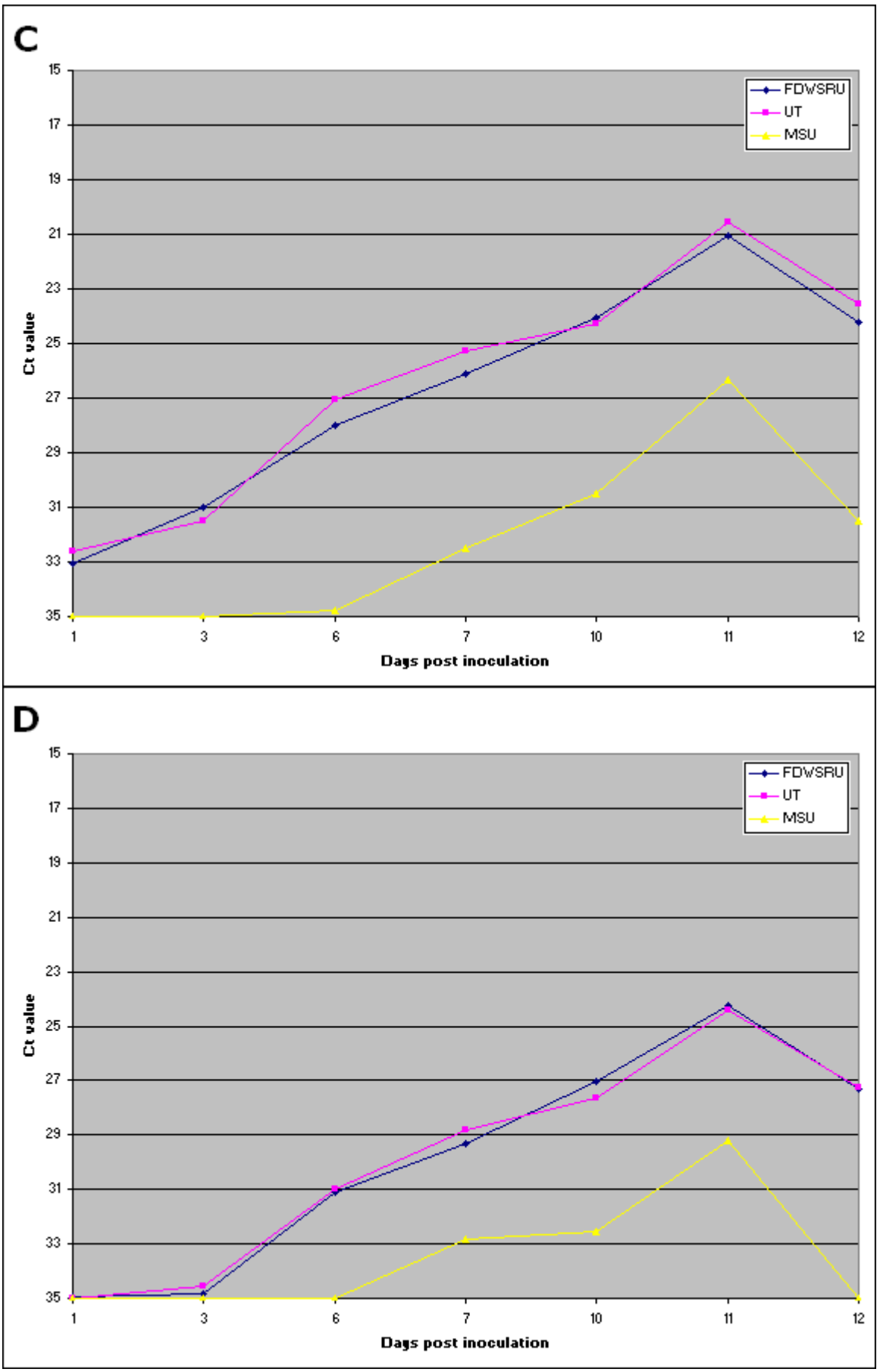
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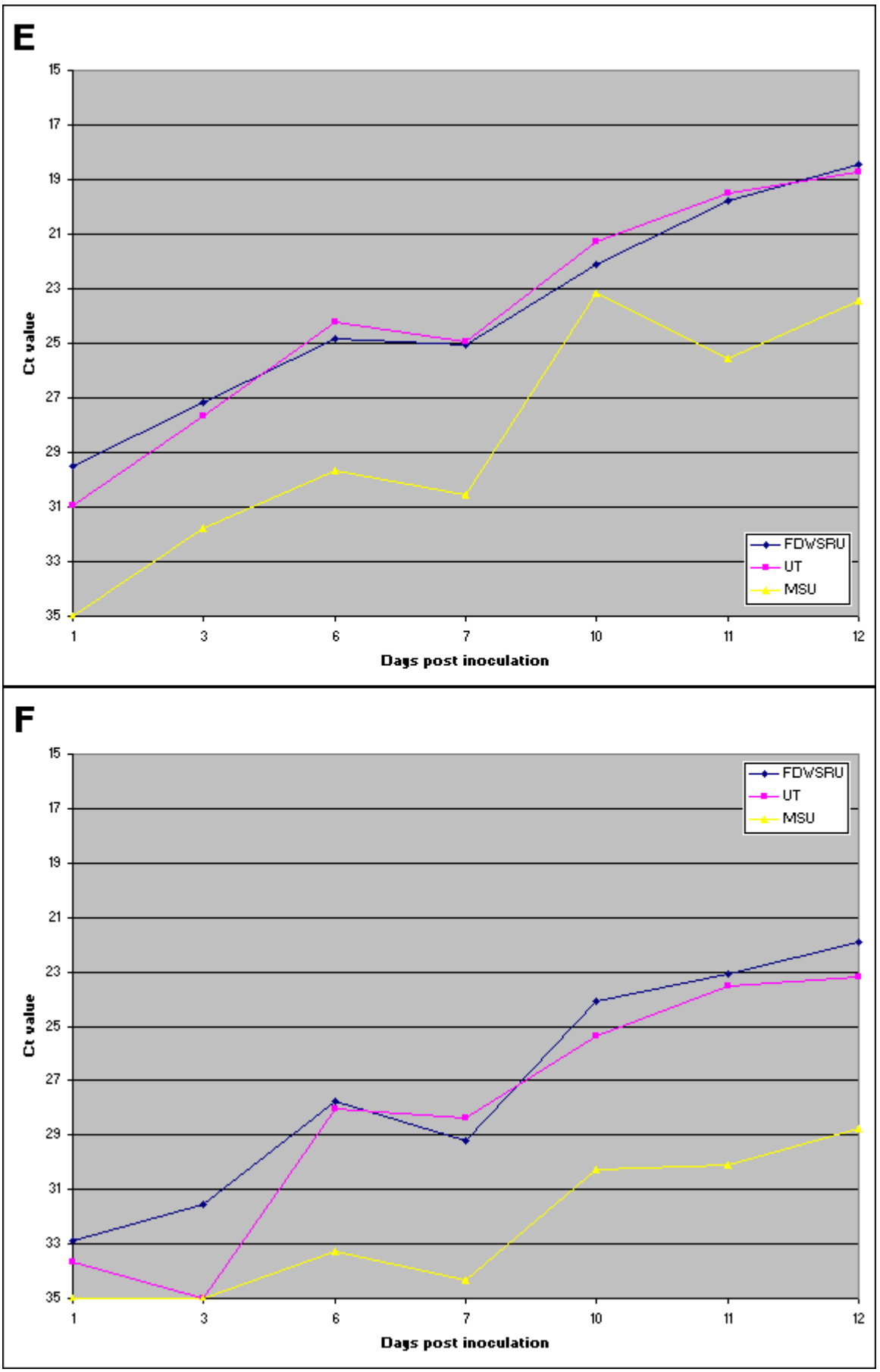
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Additional real-time PCR assays on samples from three of the four inoculum concentrations (12,500, 25,000, and 100,000 spores/ml) were performed at Cornell, KSU, MSU, UF, NPGBL, and the FDWSRU using the Smart Cycler II (Cepheid, Inc., Sunnyvale, CA). Smart Cycler reactions were performed in a total volume of 25 µl containing 300 nM of each primer and 100 nM FAM-probe. OmniMix HS lyophilized PCR Master Mix beads (Cepheid) were used at Cornell, KSU, UF, and the NPGBL, while 1X Platinum qPCR SuperMix-UDG (Invitrogen Corp., Carlsbad, CA) was used at the FDSWRU. Cycling conditions for the Smart Cycler reactions were as follows: 60°C for 2 min, 95°C for 2 min, 35 cycles of 95° C for 5 sec, and 60°C for 30 s. The Smart Cycler software was used to calculate the Ct values for each reaction. Similar to the results with the ABI system, ASR was detected at 6 dpi at all of the laboratories using DNA from plants inoculated at all three spore concentrations. The 1:2 DNA dilutions consistently produced stronger positive signal compared to the 1:20 DNA dilutions, regardless of which commercial kit was used to extract DNA (Fig. 4 and 5). The results of the real-time PCR assays are consistent among the laboratories regardless of the platform (ABI 7000, ABI 7700, or Smart Cycler) that was used, however, there appeared to better consistency among the laboratories with the Smart Cycler platform.

While both the traditional and real-time PCR assays detected ASR at 6 dpi, the real-time PCR assay is less labor intensive and more sensitive. Traditional PCR assays using DNA extracted from plants inoculated at 100,000 spores/ml yielded an obvious DNA band on agarose gels, but the PCR from DNA extracted from the plants inoculated at the lower spore concentrations produced faint bands that could easily be overlooked. However, the real-time PCR assays produced Ct values that are clearly positive at all three spore inoculum concentrations.

Fig. 4 (next 3 pages). Real-time PCR of soybean leaves infected with *Phakopsora pachyrhizi* performed using the Cepheid Smart Cycler. The vertical axis shows the cycle threshold (Ct) value (the PCR cycle when fluorescence exceeds background) that indicates a positive identification. The horizontal axis indicates the number of days after inoculation at which each sample was collected. DNA was extracted from inoculated leaves using the DNeasy Plant Mini kit (QIAGEN) and tested at two dilutions at the inoculum concentration as follows: (**A**) 1:2 DNA dilution, inoculum at 12,500 spores/ml; (**B**) 1:20 DNA dilution, inoculum at 12,500 spores/ml; (**C**) 1:2 DNA dilution, inoculum at 25,000 spores/ml; (**D**) 1:20 DNA dilution, inoculum at 25,000 spores/ml; (**E**) 1:2 DNA dilution, inoculum at 100,000 spores/ml; and (**F**) 1:20 DNA dilution, inoculum at 100,000 spores/ml.

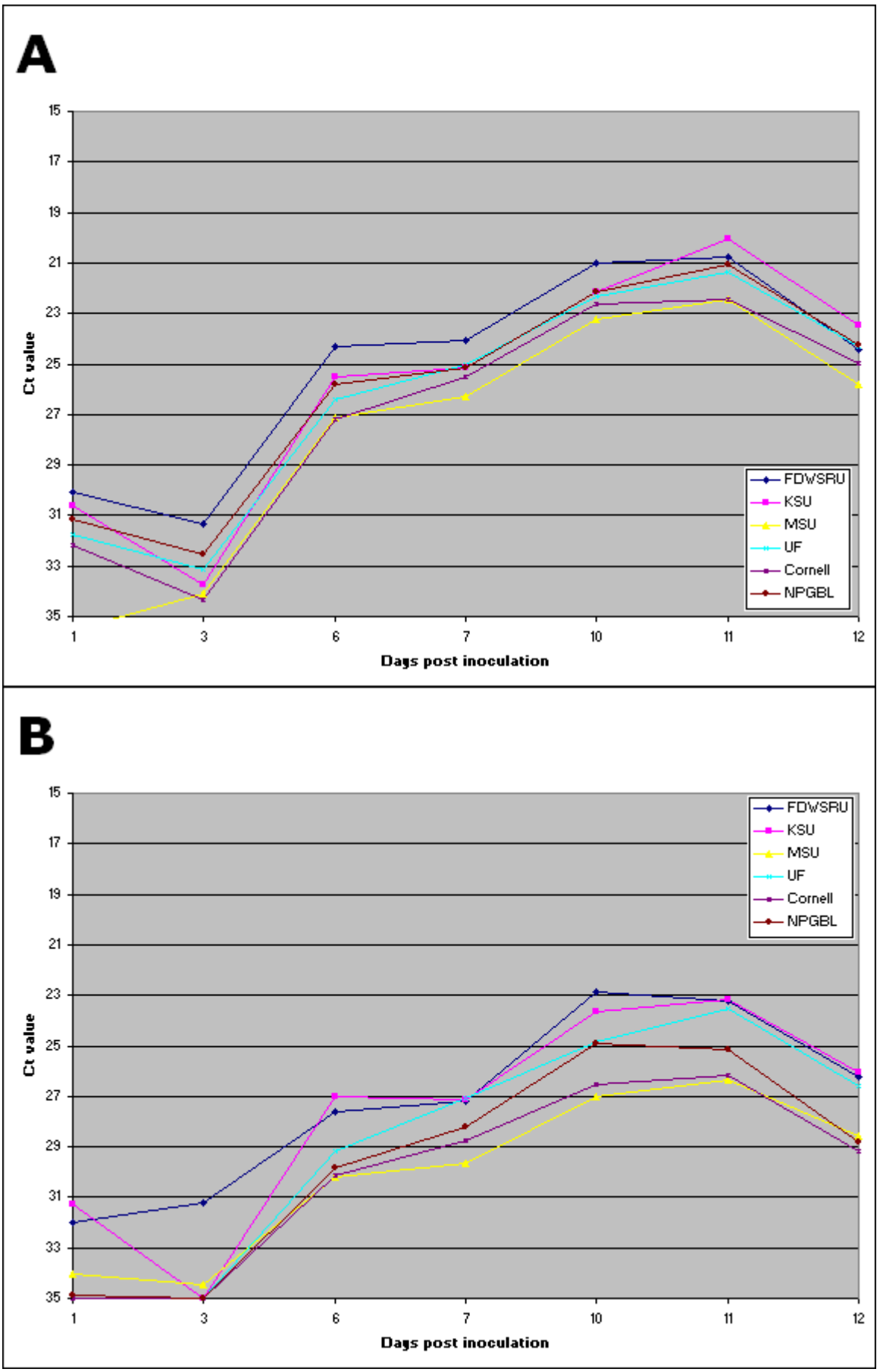
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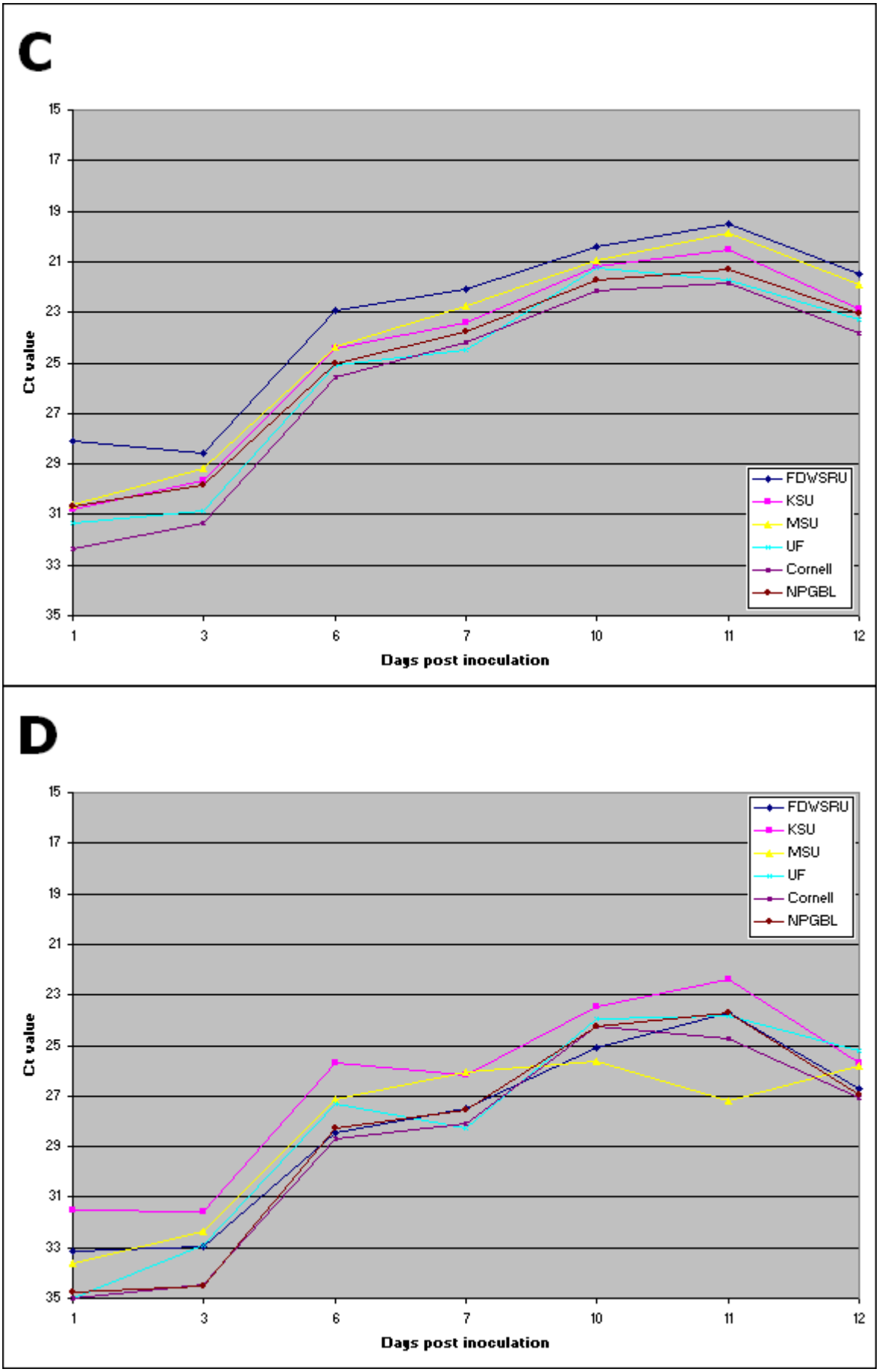
Fig. 4.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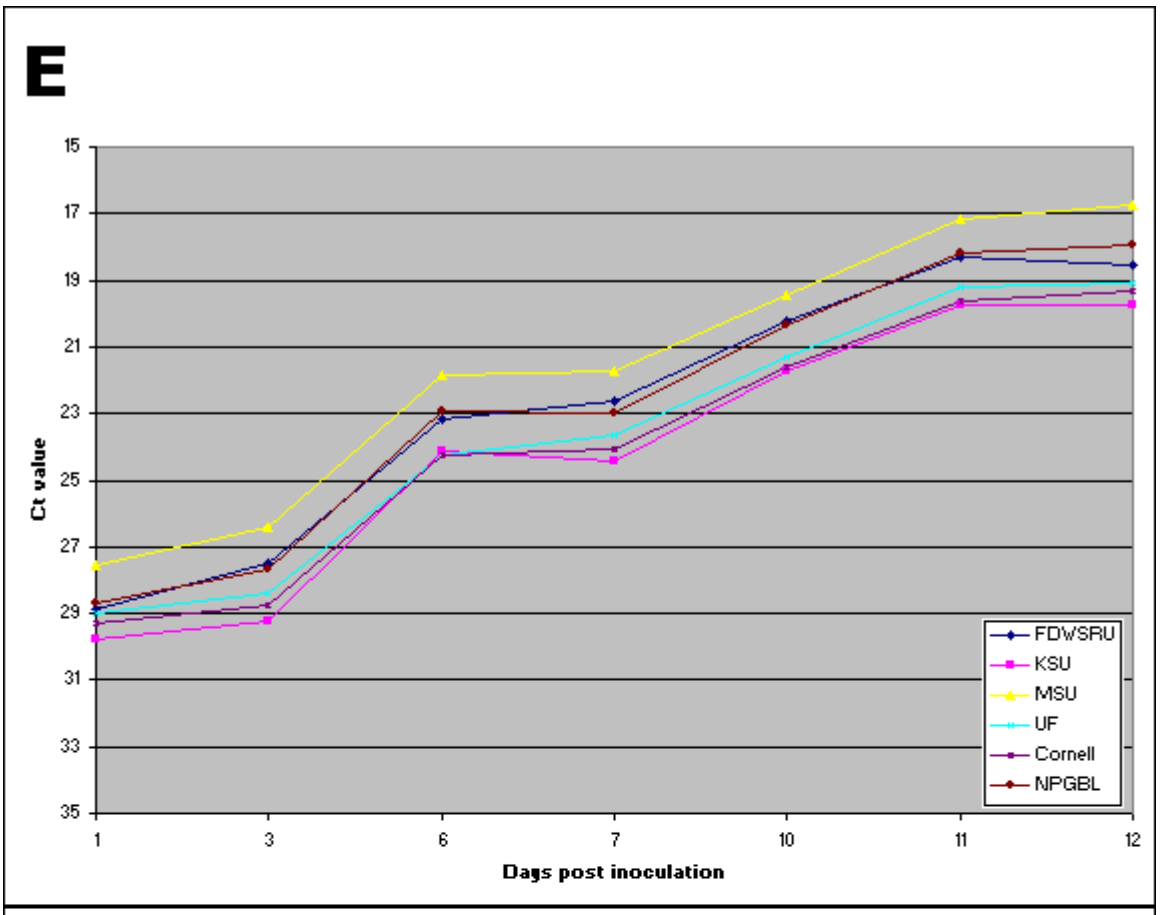
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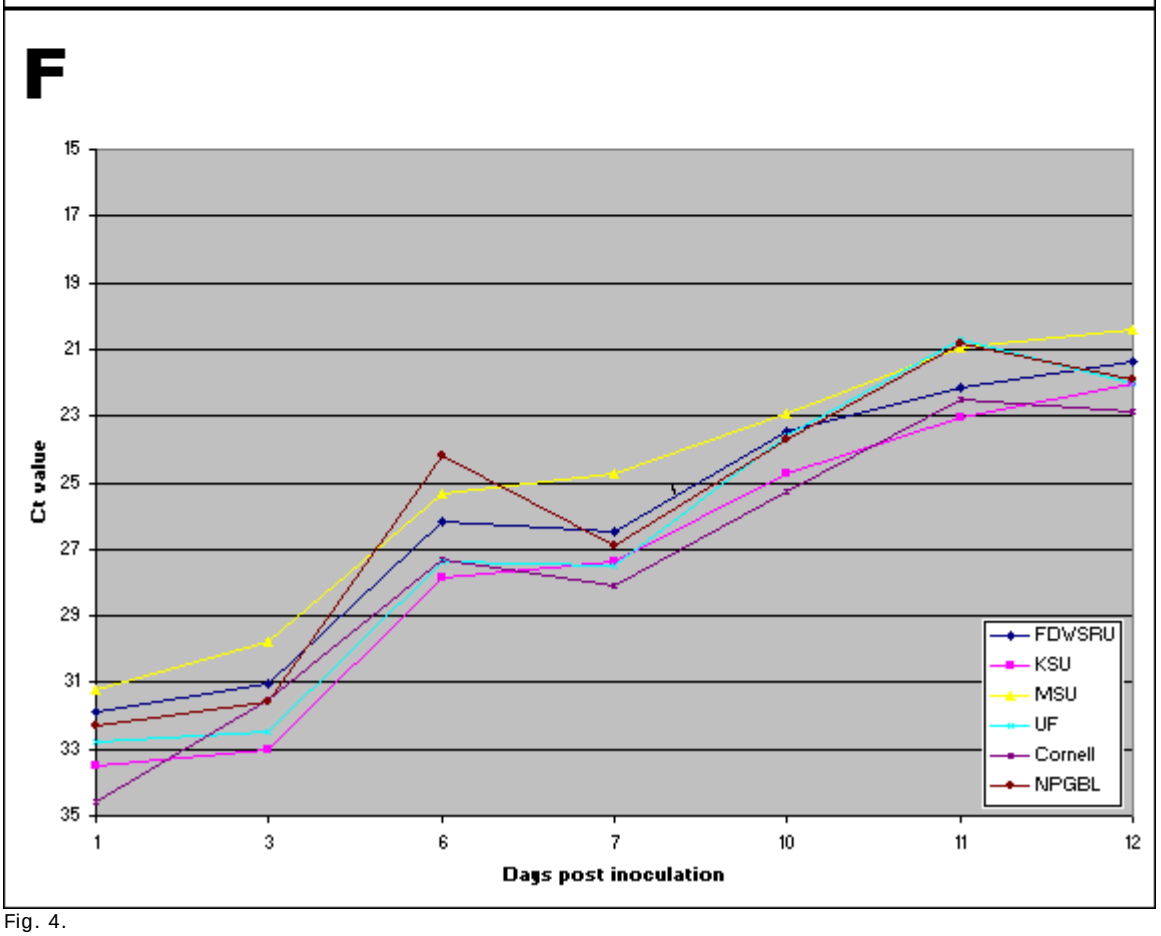
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Fig. 4.

Real-time PCR Assays from Individual Lesions

Urediniospores were suspended in sterile distilled water containing 0.01% Tween 20 at concentrations of 5,000, 10,000, and 15,000 spores/ml, and soybean plants were spray-inoculated as described previously. Nine soybean plants at the third trifoliolate stage (V3) were inoculated at each of the three spore concentrations. The plants were incubated overnight in a dew chamber at 20°C then transferred to a greenhouse and maintained under the same conditions as described. Plants were inspected daily for symptoms. When lesions first became visible under magnification at 6 dpi (Fig. 6A-C), one trifoliolate was removed from a plant and individual leaflets were examined using a dissecting scope with transmitted light. Single lesions were collected using a 1-ml pipette tip with the tip cut off to yield 3-mm leaf discs.

DNA extractions from each of twelve leaf discs containing single lesions were performed using the DNeasy Plant mini kit (QIAGEN, Inc., Valencia, CA) following the manufacturer's protocol. From each extraction, 1µl of DNA was used for testing at the FDWSRU with the Smart Cycler conditions as described above and using OmniMix HS lyophilized PCR Master Mix beads and the ASR-specific Primer and Probe Set dry beads (Cepheid). Positive PCR amplification curves demonstrate that ASR was detected in all 12 individual lesions using the Smart Cycler (Fig. 6).

Fig. 5 (next 3 pages). Real-time PCR of soybean leaves infected with *Phakopsora pachyrhizi* performed using the Cepheid Smart Cycler. The vertical axis shows the cycle threshold (Ct) value (the PCR cycle when fluorescence exceeds background) that indicates a positive identification. The horizontal axis indicates the number of days after inoculation at which each sample was collected. DNA was extracted using the QIAamp DNA Stool mini kit (QIAGEN) and tested at two dilutions at the inoculum concentration as follows: (**A**) 1:2 DNA dilution, inoculum at 12,500 spores/ml; (**B**) 1:20 DNA dilution, inoculum at 12,500 spores/ml; (**C**) 1:2 DNA dilution, inoculum at 25,000 spores/ml; (**D**) 1:20 DNA dilution, inoculum at 25,000 spores/ml; (**E**) 1:2 DNA dilution, inoculum at 100,000spores/ml; and (**F**) 1:20 DNA dilution, inoculum at 100,000 spores/ml.

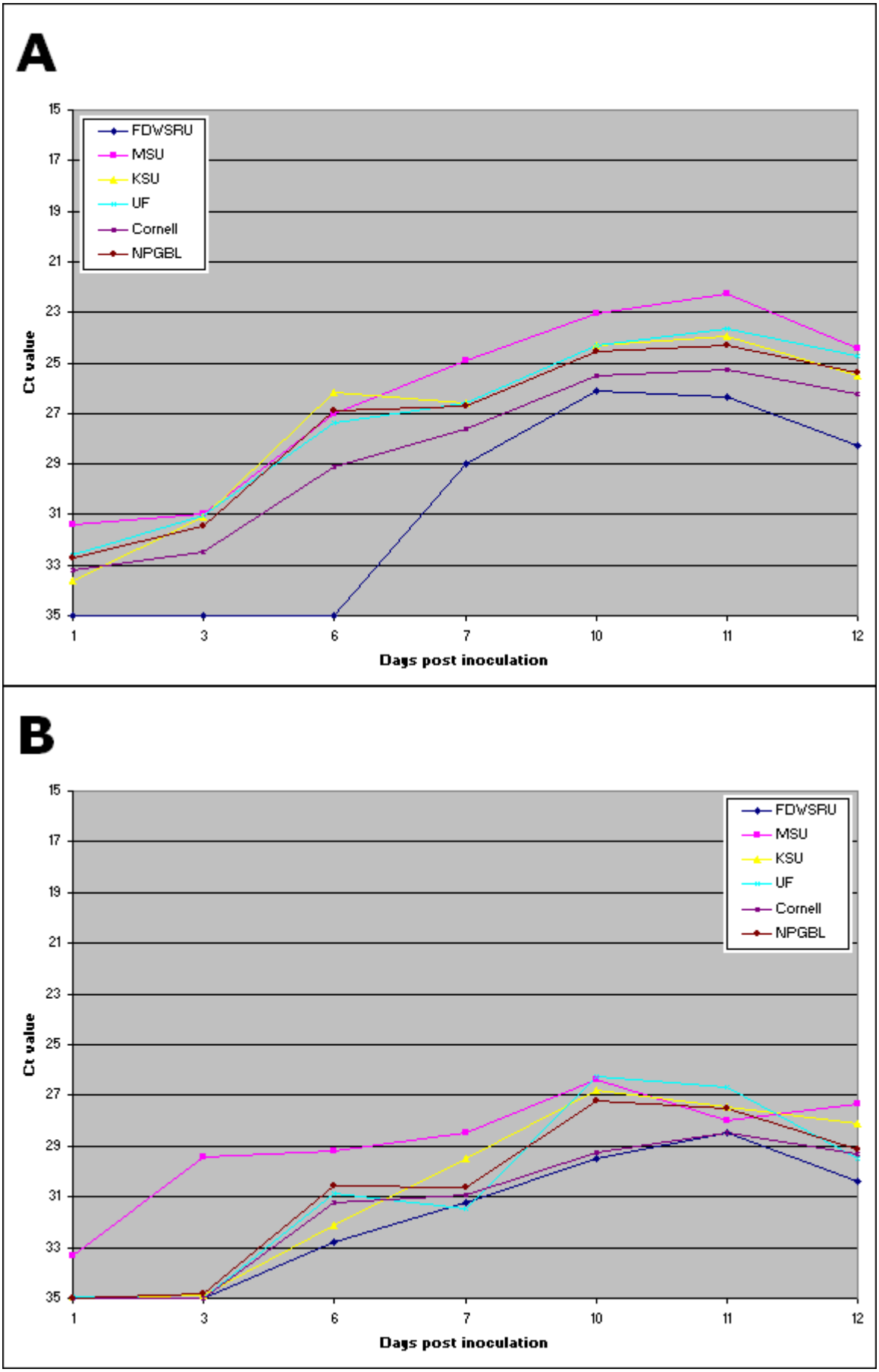
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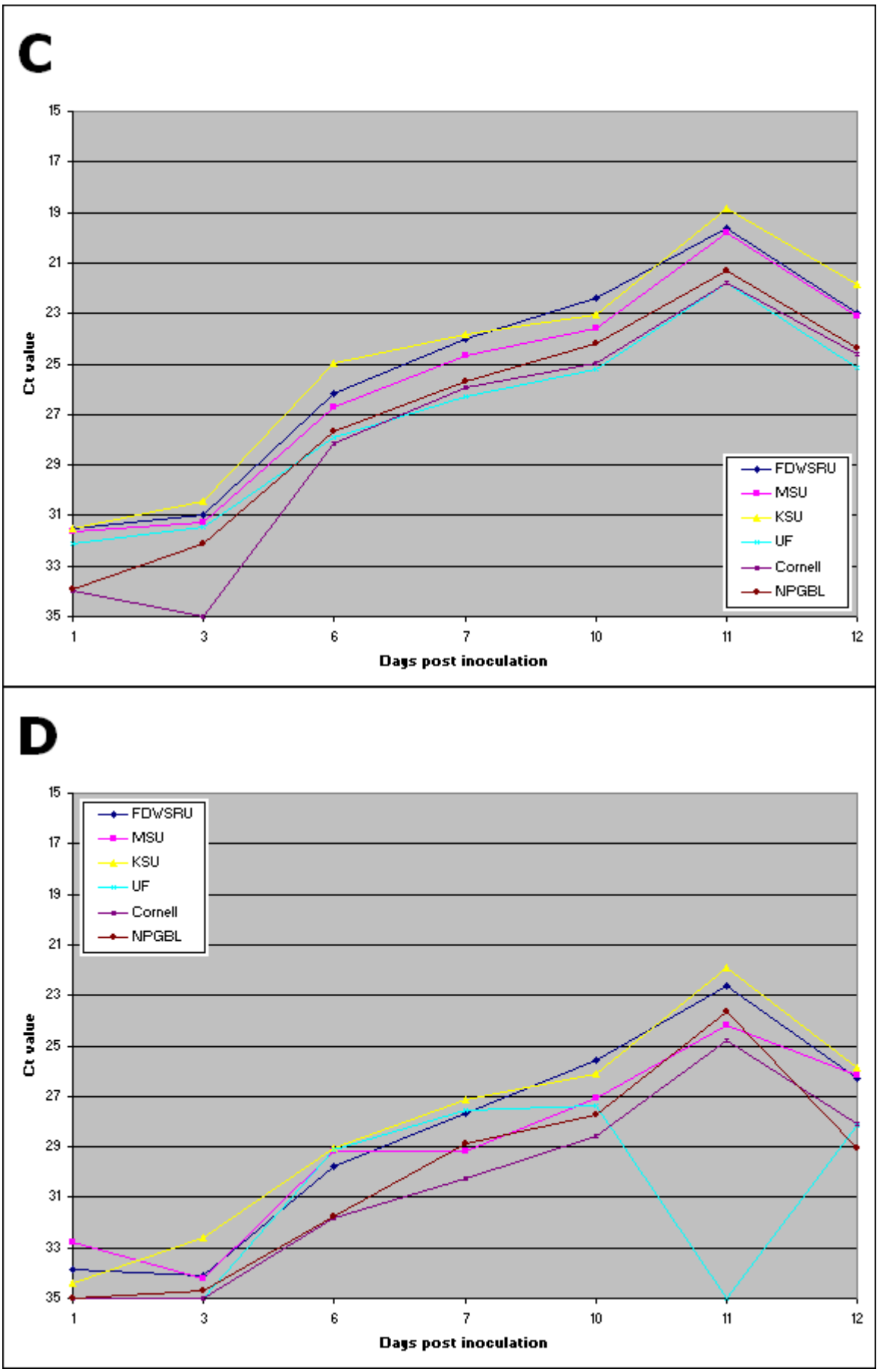
Fig. 5.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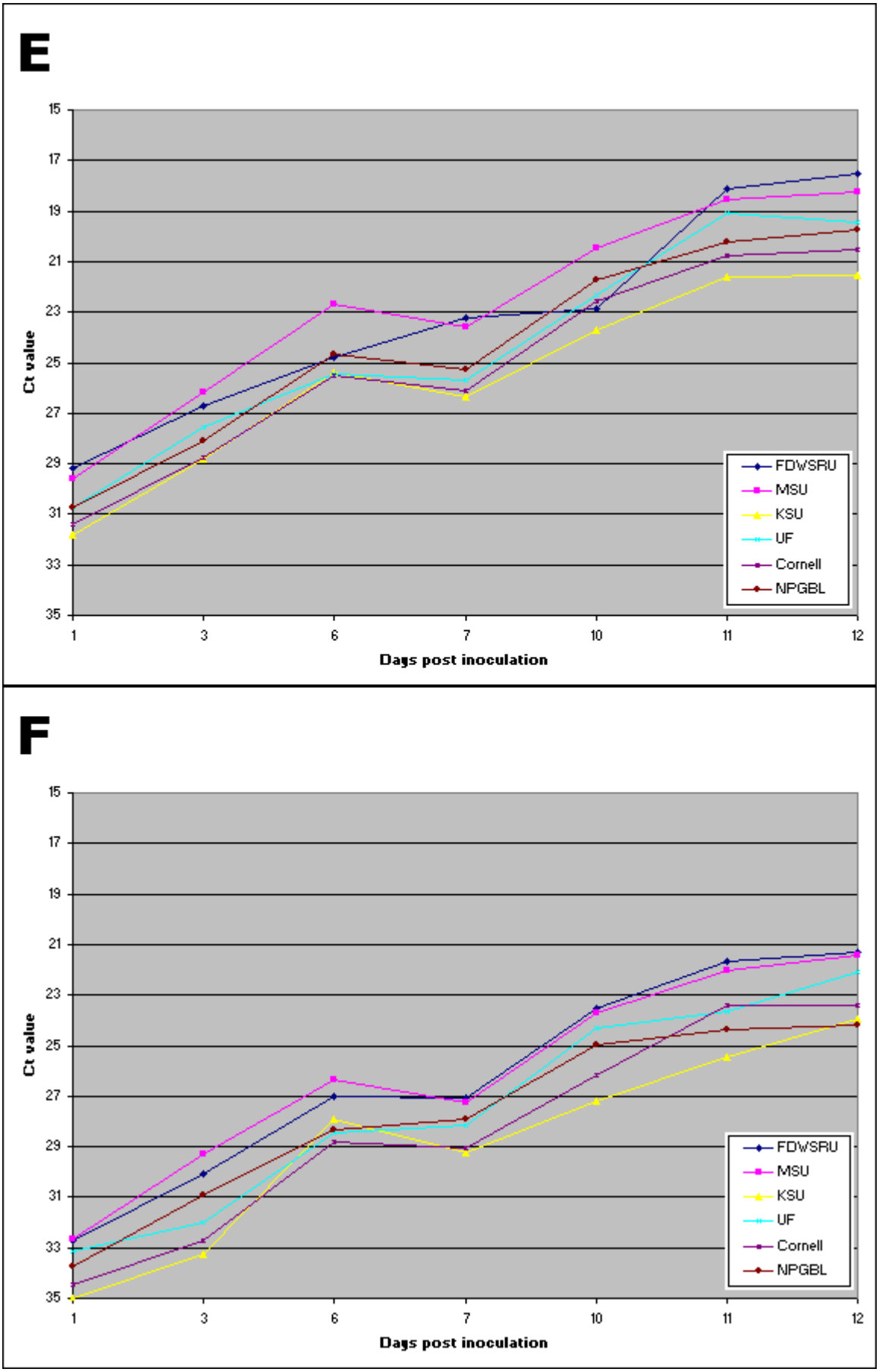
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Fig. 5.

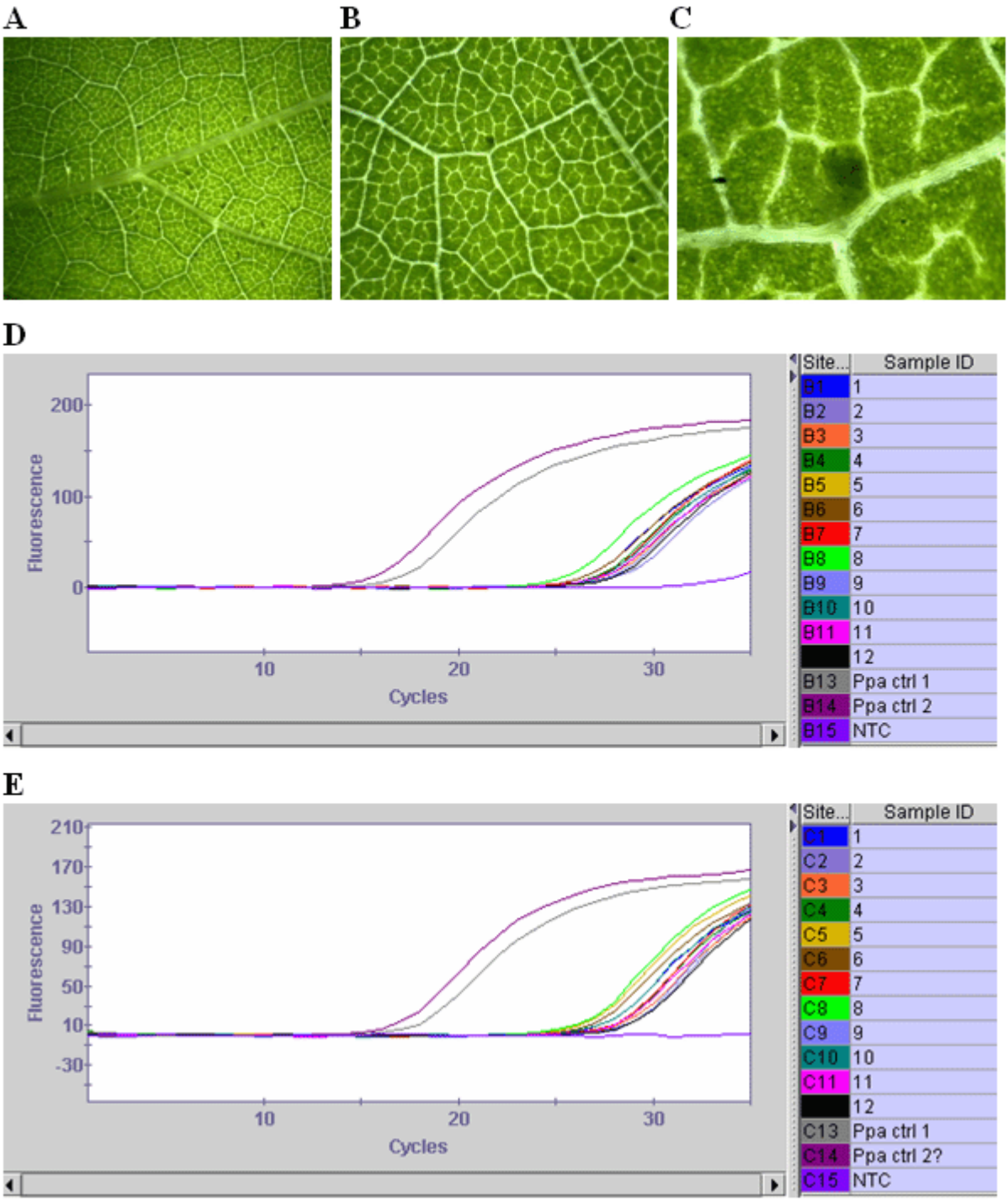

Fig. 6. Detection of *P. pachyrhizi* from single lesions collected at six days post inoculation. Rust lesions were observed using light microscopy at the following magnification: (**A**) 7x, (**B**) 15x, and (**C**) 70X. Twelve single lesions were collected from ASR infected soybean leaves and DNA extractions were performed using the DNeasy Plant mini kit (QIAGEN). From each extraction, 1 µl of DNA was used for real-time PCR assays using the Cepheid Smart Cycler with (**D**) wet chemistry or (**E**) dry bead formulations.

Implications and Practical Applications

ASR can complete multiple infection-sporulation-infection cycles within a single growing season. When the environmental conditions are optimal for ASR, the first urediniospores are produced as early as 9 days after infection, and spore production can continue for up to 3 weeks (4). The PCR tests described here can detect ASR in soybean tissue at 6 dpi, when initial disease lesions may often be observed under magnification, but well before uredinia and urediniospores are present for accurate identification and disease diagnosis.

DNA was extracted from rust infected soybean leaves with two commercially available DNA extraction kits that are readily available and easy to use. Our results were essentially the same for both kits and it is likely that any of the many different published protocols for extracting DNA from plants will work equally well.

It must be noted that the sample material used in this test was greenhouse-grown, infected under ideal conditions, and free of other diseases or pests common to soybeans grown in the field. Subsequent steps for full validation of the PCR assay with field-grown plants exposed to a range of environmental growth conditions and expressing disease over variable time scales will determine the true utility of the PCR assay as a decision making tool.

As with any diagnostic assay that requires a limited amount of sample material there are multiple decision-making stages in the sampling process that impact the outcome of the assay. ASR symptoms, especially early in the disease progression, are relatively non-specific and the choice of which leaves (e.g., old vs. new, heavily damaged vs. slightly symptomatic, widely dispersed vs. close together) to harvest for testing may not be obvious. Even if leaves harboring ASR are harvested, the material must then be sampled again, often by another person, to include lesions with enough fungal biomass to allow for positive amplification. In a situation where this PCR assay is used to screen sentinel plots for early detection of ASR the final amount of tissue that is actually tested is a tiny fraction of the total biomass in the plot. Clearly there is ample opportunity for negative PCR results where ASR is indeed present, and it is very important that guidelines for sampling leaves be developed. As for false positives, we did not record any positive reactions with any of our negative controls.

This is the first step in validation of the real-time and traditional PCR assays for *P. pachyrhizi*, and represents a major step forward in advancing the diagnostic capability of plant pathogen diagnostic laboratories to detect and identify ASR in soybean tissue well before urediniospore formation and subsequent spore dissemination occur in a leaf lesion. Our results illustrate the reproducibility of the assay among independent diagnosticians at multiple laboratories. The ASR real-time PCR assay may thus become an important decision making tool for crop consultants, producers, and Extension specialists to use in deciding when to apply fungicides when climatic conditions are favorable for ASR and/or ASR spores are detected in the local environment.

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